Eur. J. Clin. Chem. Clin. Biochem.
Vol. 32, 1994, pp. 107–111

Studies on the Concentrations of Arsenic, Selenium, Copper, Zinc and Iron in the Hair of Blackfoot Disease Patients in Different Clinical Stages

By *C. T. Wang*[1,4], *W. T. Chang*[1], *C. W. Huang*[1], *S. S. Chou*[2], *C. T. Lin*[3], *S. J. Liau*[3] and *R. T. Wang*[4]

[1] *Department of Chemistry, Chung-Yuan Christian University, Chung Li, Taiwan, Republic of China*
[2] *National Laboratories of Food and Drugs Department of Health Executive, Yuan, Taipei, Taiwan, Republic of China*
[3] *Taiwan Provincal Chia-Yi Hospital, Chia-Yi, Taiwan, Republic of China*
[4] *Department of Food Sanitation, Ta-Jen Pharmaceutical College, Ping-Tung, Taiwan, Republic of China*

(Received August 23/November 22, 1993)

Summary: Flame atomic absorption spectrophotometric methods were developed for, arsenic, selenium, copper, zinc and iron in hair samples. Data from blackfoot disease patients at five clinical stages were compared with those from healthy controls. The copper and zinc concentrations showed only slight differences in all clinical stages, which indicated the less relation to blackfoot disease.

The decrease of selenium and iron in all stages was attributed to the antagonistic effect of arsenic; arsenic increased in the first and second stages, but decreased in the later stages. The decrease of selenium and iron during the progression of the disease is thought to be due to persistence of the antagonistic effect of arsenic in the initial stages, so that very low concentrations of selenium are found in the advanced stages, despite the later decrease of arsenic. There was also a progressive decrease of iron with advance of the disease, and the later stages also showed a decrease in haemoglobin.

It was shown that arsenic is a major cause of blackfoot disease, and that it antagonises selenium and iron, which decreased in the advanced clinical stages of the disease.

Introduction

An endemic peripheral vascular disease called blackfoot disease is suffered by a large number of inhabitants on the southwest coast of Taiwan (1–6). The disease has an insidious onset, with numbness or coldness as the initial symptom, followed by progressive development of localized ulceration and subsequent gangrenous changes, giving the characteristic black coloration of dry gangrene. Most blackfoot disease patients are permanently maimed as a result of spontaneous or artifical amputation of a portion of the affected extremity (7).

High levels of arsenic, chromium, silicate, copper, iron, nickel, manganese or fluorescent compounds in drinking water from artesian wells are thought to be possible causes of blackfoot disease, with arsenic as the primary suspect (8–18). It is therefore likely that blackfoot disease patients with more advanced clinical symptoms will show increased levels of arsenic, copper, zinc and iron in their blood and hair, but our preliminary results (19) showed a decrease of blood arsenic, selenium, zinc, and iron in the advanced stages of the disease. In order to assess this situation we analysed the levels of arsenic, selenium, copper, zinc and iron in hair samples from blackfoot disease patients at five different clinical stages. Our results indicated antagonistic effects between arsenic, selenium and iron.

Materials and Methods

Hair samples

One hundred and sixty-eight hair samples of blackfoot disease patients at five clinical stages according to the criteria set by Drs.

U. C. Huang and *C. T. Lin* (as shown in tab. 1), and 50 hair samples of persons without known history of exposure to arsenic were used. There was an even distribution of sex and age (tab. 2).

Chemicals and biological standards

Suprapur grade reagents of E. Merck and high purity water (18 MΩ) were used. Stock solutions containing 1000 mg/l of iron, zinc, copper, selenium and arsenic and working standard solutions were prepared from Merck Tritrisol standards by diluting with the high purity water. Containers made of quartz, teflon, or polypropylene were used throughout. They were immersed in 8 mol/l HNO_3 overnight and washed with several changes of distilled water. Human hair No. 5 from the National Institute for Environmental Studies (Japan) was used as a standard.

Analytical methods

Atomic absorption spectrophotometer model Z-8000 and its accessory hydride formation system HFS-2 from Hitachi, Japan were used for determining trace metals. A CEM-MD 2000 Microwave digester (U. S. A.) was used for sample digestion.

Hair (0.2000 ± 0.0200 g) was weighed into a digestion vessel, followed by 10 ml of conc. HNO_3. The power output of the digester was maintained at 30% for 5 min and at 0% for 10 min. After adding 10 ml water, the digestion was continued at 40% power for 25 min and at 0% for 10 min. Finally, 2 ml of H_2O_2 was added and the power kept at 65% until a colourless solution was obtained (about 12 min).

The digest was diluted to 50 ml with the high purity water. Aliquots were taken for the ordinary (iron, zinc and copper) and hydride (arsenic and selenium) modes of the atomic absorption spectrophotometer (20–24).

Tab. 1 The different stages of blackfoot disease as classified in Chia-Yi Hospital, Taiwan.

States	Symptom
Zero	Assumed to be present in residents of endemic area without evidence of disease.
First	Coldness, numbness and pain.
Second	Evidence of slight ulceration and gangrene.
Third	Evidence of strict ulceration and gangrene.
Fourth	Evidence of gangrenous change of the affected extremity. Spontaneous or artificial amputation of foot or hand.

Tab. 2 The distribution of specimens.

	Healthy controls	Stage of blackfoot disease				
		Zero stage	1st stage	2nd stage	3rd stage	4th stage
Male	29	20	32	18	10	21
Female	21	21	26	7	8	10

Note: The ages of the patients and healthy controls were about 65 ± 10 years.

Results and Discussion

Data accuracy and analyte recovery

To monitor data quality, human hair standards were analysed. As shown in table 3, the best recovery and accuracy (CV%) for the human hair standard were respectively 100% and less than 6.0% for iron, 97% and less than 5.4% for zinc, and 95% and less than 4.5% for copper. But selenium showed a high CV% of 7.8% with a recovery of 92%.

Recoveries of arsenic added to aqueous digests of hair from healthy controls and blackfoot disease patients in the first clinical stage are compared in table 4. Samples from blackfoot disease patients gave a higher CV (16.2%) and recovery (92.0%) than those of healthy controls (15.2 and 90.1%, respectively).

Recovery and standard curves

Standard and recovery curves of iron, zinc, copper, selenium and arsenic were obtained by analysing healthy control hair samples. To 1 ml of the sample was added 0.4, 1, 2, 4 or 10 µg of iron; 0.2, 0.4, 1 or 2 µg of zinc and copper; and 2, 4, 10 or 20 ng of selenium and arsenic.

The results are shown in figures 1 and 2. Good linear relationships for concentration were obtained. Good recoveries of 100, 97 and 95% were obtained for iron, zinc and copper, respectively. Poorer recoveries of 90 and 92% were found for arsenic and selenium, respectively, owing to their volatility.

Analyses of hair samples

Table 5 shows the analytical results of hair samples obtained from the healthy controls and blackfoot disease patients without discrimination of clinical stages. It was shown that the copper, zinc and iron contents of hair from blackfoot disease patients did not differ greatly

Tab. 3 Recoveries of analysis of National Institute for Environmental Studies (Japan-Environment Agency) human hair No. 5 by hydride flame atomic absorption (Se) and flame atomic absorption (Fe, Cu, Zn) methods (n = 6).

No. 5 Human Hair	Fe	Cu	Zn	Se
Measured value (mg/kg)	2.55 ± 0.15	15.5 ± 0.7	165 ± 9.0	1.28 ± 0.10
CV (%)	6.0	4.5	5.4	7.8
Certified value (mg/kg)	2.55 ± 0.10	16.3 ± 1.2	169 ± 10	1.4
CV (%)	3.9	7.3	5.9	
Recovery (%)	100	95	97	92

Tab. 4 Recoveries of arsenic added to aqueous digest of hair from healthy controls blackfoot disease patients in the first stage of the disease (n = 6).

Aqueous digest solutions from	No. of specimens	As added (μg/l)	As recovered (μg/l)	CV (%)	Recovery (%)
Healthy controls	8	100	90.0 ± 13.6	15.2	90.0
Blackfoot disease patients	8	100	92.0 ± 14.9	16.2	92.0

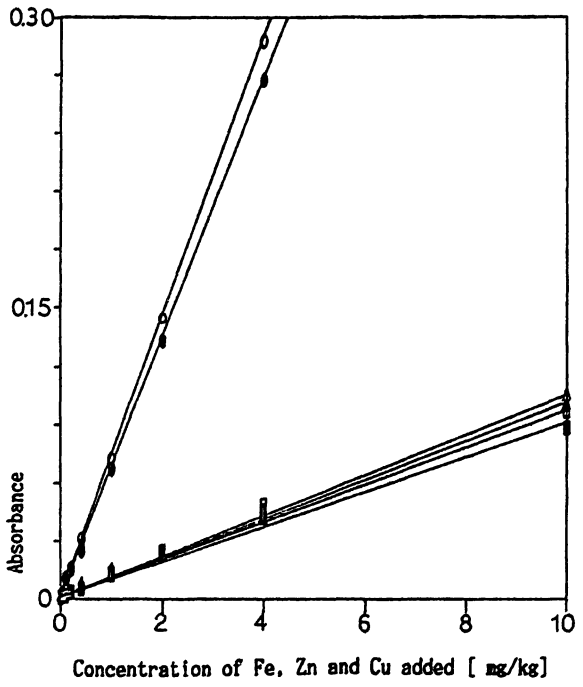

Fig. 1 Standard curves for the assay the iron, copper and zinc.
△ Fe in purified water ▲ Fe in normal person hair
□ Cu in purified water ■ Cu in normal person hair
○ Zn in purified water ● Zn in normal person hair

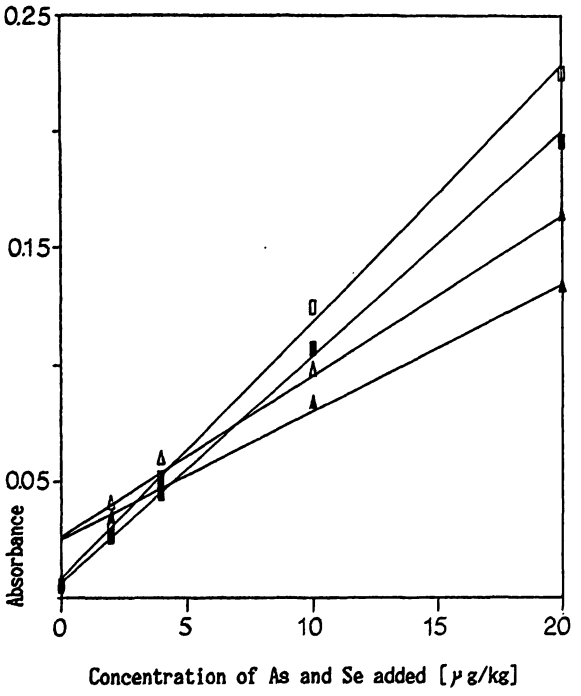

Fig. 2 Standard curves for the assay the arsenic and selenium.
△ As in purified water ▲ As in normal person hair
□ Se in purified water ■ Se in normal person hair

from those of healthy controls; they fall within a 10% deviation (copper 0.16 ± 0.10 and 0.15 ± 0.07 mmol/kg; iron 0.86 ± 0.49 and 0.85 ± 0.50 mmol/kg and zinc 2.22 ± 1.31 and 2.02 ± 0.93 mmol/kg), but arsenic and selenium showed significant differences, with deviations of + 50 and −15%, respectively (arsenic 2.40 ± 1.60 and 3.60 ± 2.13 μmol/kg; selenium 1.89 ± 0.88 and 1.51 ± 0.75 μmol/kg). The hair samples from blackfoot disease patients had lower selenium but higher arsenic contents than those of healthy controls.

Arsenic and selenium concentrations in the hair of blackfoot disease patients in different clinical stages, who were living in high endemic areas, were compared with those living in low endemic areas (tab. 6). Samples from blackfoot disease patients living in high endemic areas showed a significantly higher value (4.40 ± 2.13 μmol/kg) for arsenic, but a lower value (1.51 ± 0.81 μmol/kg) for selenium, compared with the samples from low endemic areas (3.06 ± 1.73 μmol/kg for arsenic, 2.00 ± 1.07 μmol/kg for selenium). The differences amounted to + 30% for arsenic and −25% for selenium. There is therefore a positive correlation between hair arsenic and selenium and the onset of blackfoot disease.

Antagonism of selenium by arsenic

Table 7 shows the analytical results for hair specimens from blackfoot disease patients at the different clinical stages. It shows that the differences between hair copper and zinc in blackfoot disease patients and healthy controls were always within ± 10%. But arsenic, selenium and iron showed larger differences of more than ± 15%. The copper concentrations for blackfoot disease patients and healthy controls showed an average value of 0.15 ± 0.08 mmol/kg. Therefore hair copper seems to have little or no effect on hair arsenic. Zinc shows a slight decrease with the clinical advance of the disease (from 20.6 to 18.2 mmol/kg).

The antagonism of hair selenium by arsenic is relatively pronounced; as the clinical stages progress from zero to the second, the hair arsenic increases significantly from 2.66 to 6.13 μmol/kg and selenium decreases from 1.64

Tab. 5 Comparisons of hair arsenic, selenium, copper, zinc and iron contents in blackfoot disease patients and healthy controls.

	Concentration		Deviation between healthy controls and blackfoot disease patients	
	Healthy controls (n = 50)	Blackfoot disease patients (n = 168)	(%)	P value (t-test)
Arsenic (μmol/kg)	2.40 ± 1.60	3.60 ± 2.13	+50	P < 0.001
CV (%)	66	59		
Selenium (μmol/kg)	1.89 ± 0.88	1.51 ± 0.75	−15	P < 0.001
CV (%)	51	50		
Copper (mmol/kg)	0.16 ± 0.10	0.15 ± 0.07	−10	P < 0.025
CV (%)	60	48		
Zinc (mmol/kg)	2.22 ± 1.31	2.02 ± 0.93	− 9	P < 0.001
CV (%)	59	46		
Iron (mmol/kg)	0.86 ± 0.49	0.85 ±0.50	− 1	P > 0.05
CV (%)	57	59		

Tab. 6 Comparisons of hair arsenic and selenium concentrations of blackfoot disease patients from high and low endemic areas.

	High endemic area patients (μmol/kg) (n = 101)	CV (%)	Low endemic area patients (μmol/kg) (n = 20)	CV (%)	Deviation between the high and low endemic patients	
					(%)	P values (t-test)
Arsenic	4.40 ± 2.13	48	3.06 ± 1.73	56	+30	P < 0.001
Selenium	1.51 ± 0.81	54	2.00 ± 1.07	53	−27	P < 0.001

Tab. 7 Comparisons of hair arsenic, selenium, copper, zinc, and iron in different clinical stages of blackfoot disease patients and healthy controls.

			Clinical stages of blackfoot disease				
		Healthy controls	Zero stage	1st stage	2nd stage	3rd stage	4th stage
Arsenic	(μmol/kg)	2.40	2.66	4.67	6.13	5.73	3.06
Selenium	(μmol/kg)	1.90	1.64	1.64	1.52	1.45	1.39
Copper	(mmol/kg)	1.66	1.53	1.60	1.37	1.55	1.33
Zinc	(mmol/kg)	22.2	20.6	20.6	20.5	18.9	18.2
Iron	(mmol/kg)	8.60	10.03	9.14	8.60	7.16	5.37

to 1.52 μmol/kg. At the third and fourth stages, the level of selenium is maintained at about 1.39 μmol/kg, exerting a continual antagonistic effect, which causes the hair arsenic content to decrease to 3.06 μmol/kg. This is the reason, we believe, why some reports describe lower hair arsenic values in blackfoot disease patients and yet most reports emphasize arsenic as a major causative agent of the disease, based on the results of drinking water analyses. This antagonistic effect is not only of academic interest and worthy of further study but also important in the clinical treatments of blackfoot disease patients.

The hair iron content of blackfoot disease patients decreases as the clinical stages progress from zero to the fourth, with values changing from 10.03 to 5.37 mmol/kg. Two reasons may be offered for this decrease: the antagonism of iron by arsenic, and the loss of haemoglobin in the later stages. The iron status of blackfoot disease patients is also worthy of further study.

Acknowledgement

The authors would like to express their appreciation to the Paolyta Co. Ltd. for the financial support of this work, and special thanks are due to the consultant Mr. *Chen*.

References

1. Tseng, W. P. (1973) The natural history of blackfoot disease. J. Formosan Med. Assoc. *72*, 11–24.
2. Yeh, S. & How, S. W. (1963) A pathological study in blackfoot disease in Taiwan. Reports, Institute of Pathology, National Taiwan University. No. *14*, 25–73.
3. Tsen, W. P., Chen, W. Y. & Sung, J. L. (1961) A clinical study of blackfoot disease in Taiwan, an endemic pheripheral vascular disease. Memoirs of College of Medicine of the National Taiwan Univ. *7*, 1–17.
4. Chen, K. P. & Wu, H. Y. (1962) Epidemiologic studies on blackfoot disease in Taiwan. A study of sources of drinking water in relation to the disease. J. Formosan Med. Assoc. *61*, 611–618.
5. Tseng, W. P. (1968) Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. J. Nat. Cancer Inst. *40*, 453–463.
6. Chen, K. P. (1962) Epidemiologic studies on blackfoot disease in Taiwan. 3. Physiochemical characteristics of drinking water in endemic blackfoot disease area. Memoirs of College of Medicine of the National Taiwan Univ. *8*, 115–129.
7. Lo, M. C., Hsen, Y. C. & Lin, B. K: (1977) The second report on investigation of arsenic content in underground water in Taiwan province. Taipei. Provincial Institute of Environmental Sanitation.
8. Lu, F. J. (1978) Study on fluorescent compounds in drinking water of endemic areas for blackfoot disease and reinvestigation of causes of blackfoot disease. National Science Council Monthly *6*, 388–402.
9. Lu, F. J. & Liu, T. M. (1986) Fluorescent compounds in drinking water of blackfoot disease endemic areas animal experimental model. J. Formosan Med. Assoc. *85*, 352–358.
10. Lu, F. J. (1988) Fluorescent humic substances and endemic disease in south-western Taiwan. J. Chinese Biochemical Society *17*, 57–74.
11. Wu, H. Y. & Chen, K. P. (1961) Epidemiologic studies on blackfoot disease. 1. Prevalence and incidence of disease by age, sex, year, population, and geographic distribution. Memoirs of College of Medicine of the National Taiwan Univ. *7*, 33.
12. Tseng, W. P. (1970) Prognosis of blackfoot disease: A ten year follow-up study. J. Formosan Med. Assoc. *69*, 1–21.
13. Prasad, A. S. (1979) Clinical, biochemical and pharmacological role of zinc. Ann. Rev. Pharmacol. Toxicol. *20*, 393–426.
14. Hsieh, H. S. & Ganther, H. E. (1977) Biosynthesis of dimethyl selenide from sodium selenite in rat liver and kidney cell-free system. Biochim. Biophys. Acta *497*, 205–217.
15. Lin, S. M., Chiang, C. H. & Yang, M. H. (1985) Arsenic concentration in the urine and blood of patients with blackfoot disease and bowen's disease. Biol. Trace Element Res. *8*, 11–19.
16. Keshan Disease Research Group (1979) Higher risked of the keshan disease with the lower concentration of food selenium. Clin. Med. J. *92*, 471–482.
17. Magos, L. & Webb, M. (1980) The interactions of selenium with cadmium and mercury CRC. Crit. Rev. Toxicol. *8*, 1–41.
18. Chen, X. (1980) Studies the selenium concentration in blood of the keshan disease patients Biol. Trace Element Res. *2*, 91–107.
19. Wang, C. T., Huang, C. W., Chou, S. S., Lin, C. T., Liau, S. W. & Wang, R. T. (1993) Studies on the concentration of arsenic, selenium, copper, zinc and iron in the blood of blackfoot disease patients in different clinical stages. Eur. J. Clin. Chem. Clin. Biochem. *31*, 759–763.
20. Laker, M. (1964) On determining trace element levels in man: The uses of blood and hair. Lancet *2*, 260–262.
21. Liebscher, K. & Smith, H. (1968) Essential and nonessential trace elements, determining whether an element, is essential or nonessential in human tissue. Arch. Environ. Health *17*, 881–890.
22. Heydorn, K. (1970) Environmental variation of arsenic levels in human blood determined by neutron activation analysis. Clin. Chim. Acta *28*, 349–357.
23. Lin, S. M., Chiang, C. H. & Yang, M. H. (1985) Determination of arsenic in urine and whole blood by hydride atomic absorption spectrophotometry. Koah-siung, J. Med. Sci. *1*, 105–112.
24. Lin, S. M. (1986) Diagnostic usefulness of trace arsenic in human urine, whole blood, hair and fingernails. Koah-siung, J. Med. Sci. *2*, 100–113.

C. T. Wang
1F, No. 1, Aly. 61, Ln. 114, Sec. 7
Chung Shan N. Road,
Taipei, Taiwan
Republic of China